

- PAGE) in nonreducing conditions. After electroblotting, the PVDF membrane (Millipore) was probed overnight with recombinant E2 ($1 \mu\text{g/ml}$) at room temperature. Incubation with an mAb to E2 (clone 291A2) was followed by chemiluminescent detection with a peroxidase-conjugated polyclonal anti-mouse IgG (Amersham).
14. Polystyrene beads ($1/4$ -inch diameter) (Pierce) were coated overnight with purified EC2 recombinant protein in citrate buffer (pH 4) at room temperature. After saturation for 1 hour with 2% bovine serum albumin in 50 mM Tris-Cl (pH 8), 1 mM EDTA, and 100 mM NaCl (TEN) buffer, each bead was incubated at 37°C for 2 hours in 200 μl of TEN-diluted infectious chimp plasma containing 5×10^5 HCV RNA molecules. For inhibition experiments, the EC2-coated polystyrene beads were incubated with purified mAbs (50 $\mu\text{g/ml}$) for 1 hour at room temperature before incubation with the virus. Each bead was washed five times with 15 ml of TEN buffer in an automated washer (Abbot, Wiesbaden, Germany), and viral RNA was extracted with the Viral Extraction Kit (Qiagen). RNA (8 ml) was reverse transcribed at 42°C for 90 min in 20 ml of buffer A (Taq Man; Perkin-Elmer) containing 100 pmol of the HCV antisense primer CCGTTCGCCAGACCACTATG, 40 U of RNasin (ribonuclease inhibitor) (Promega, Madison, WI), deoxynucleoside triphosphates (dNTPs) (250 μM), MgCl_2 (25 mM), and 10 U of Moloney murine leukemia virus reverse transcriptase (Boehringer). cDNA (20 μl) was amplified with a Perkin-Elmer ABI 7700 Sequence Detection System (45 cycles) in 50 μl of buffer A containing 100 pmol of the HCV sense primer TCTTCACGCAGAAAGCGTCTA, 5 pmol of the fluorescent detection probe 5'-(FAM)TGAGTGTCTGCGAGCTCCAGGA(TAMRA) (FAM is carboxyfluorescein amino-modified oligo and TAMRA is tetramethylrhodamine amino-modified oligo), dNTPs (300 μM), and 1.25 U of Taq Gold (Perkin-Elmer). All reactions were quantified with HCV (genotype 1a)-infected plasma (branched DNA titer of 30 meq/ml) to generate a standard curve. Sequence Detector Software from Perkin-Elmer has been described previously (25). To evaluate virus binding to cells, we made human CD81⁺ mouse stable transfectants (NIH 3T3) that bound E2. However, we consistently failed to measure substantial virus attachment to the cell surface by PCR because of high background inherent to the technique.
 15. In our assay, we captured only enveloped RNA molecules. The highest available concentration of human TRX-EC2 for coating beads was 100 $\mu\text{g/ml}$. At this concentration, about 7% of HCV input was bound by the beads. Using TRX-EC2 (100 $\mu\text{g/ml}$) and increasing numbers of beads, we captured about 10% of the HCV input, in terms of RNA molecules, further demonstrating that HCV binding is dependent on the CD81 concentration. Moreover, our experience with antibody (from mouse, chimp, or human)-coated beads has shown that the percentage of HCV that can be captured is negligible, further proving that CD81 is indeed a very effective binder of HCV particles.
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30. We thank C. Moretto for a contribution at the early stages of this project; S. Nuti and G. Saletti for help in

flow cytometry; J. Kansopon, D. Chien, Q.-L. Choo, S. Coates, K. Crawford, K. Berger, C. Dong, D. Piccoli, R. La Gaetana, and F. Masciopinto for technical help and reagents; G. Corsi for artwork; and R. Rappuoli, G. Del Giudice, L. Galli-Stampino, and N. Valiante for critical reading of the manuscript. We also thank D. Slade (Pharmacia and Upjohn) for providing the fluorescent detection probe.

22 June 1998; accepted 6 October

TNF- α Induction of CD44-Mediated Leukocyte Adhesion by Sulfation

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Regulation of cell adhesion is important for immune system function. CD44 is a tightly regulated cell adhesion molecule present on leukocytes and implicated in their attachment to endothelium during an inflammatory immune response. The proinflammatory cytokine tumor necrosis factor- α , but not interferon- γ , was found to convert CD44 from its inactive, nonbinding form to its active form by inducing the sulfation of CD44. This posttranslational modification was required for CD44-mediated binding to the extracellular matrix component hyaluronan and to vascular endothelial cells. Sulfation is thus a potential means of regulating CD44-mediated leukocyte adhesion at inflammatory sites.

During an immune response, activated leukocytes leave the circulation and enter the tissues. Leukocyte migration and extravasation is a multistep process involving the rolling and adhesion of leukocytes to the endothelium and their subsequent diapedesis to the inflammatory site (1). Activated T cells can bind the extracellular matrix component hyaluronan, and this

CD44-mediated interaction has been implicated in the rolling and extravasation of lymphocytes at inflammatory sites (2). CD44 is normally present on leukocytes in an inactive state that cannot bind hyaluronan but can be converted to an active state upon appropriate stimulation, such as activation by antigen or cytokines (3, 4). However, the molecular mechanism for this conversion is poorly understood. The binding ability of CD44 has been shown to be affected by three types of posttranslational modification: N- and O-linked glycosylation and glycosaminoglycan addition (4, 5). Here, we show that sulfation is an additional posttranslational mechanism that can convert inactive CD44 to its active, adhesive form and that this mechanism is induced by the proinflammatory cytokine tumor necrosis factor- α (TNF- α).

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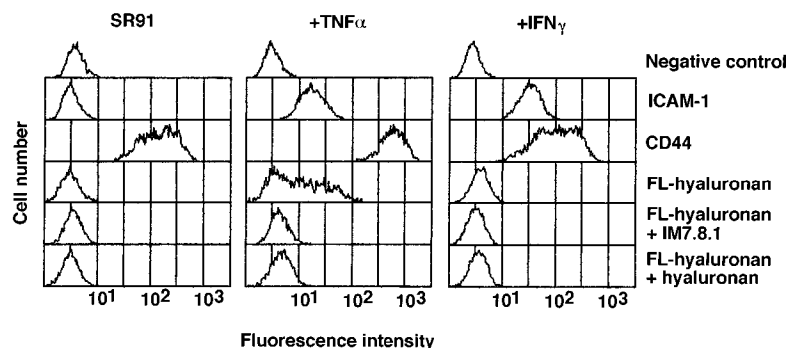


Fig. 1. Expression of CD44 and ICAM-1 and FL-hyaluronan binding ability on untreated, TNF- α -treated, or IFN- γ -treated SR91 cells by flow cytometry. Cells were preincubated with the CD44 mAb IM7.8.1, or with unlabeled hyaluronan, to block binding. Unlabeled SR91 cells were the negative control.

During an inflammatory response, cytokines, including TNF- α , stimulate leukocyte-endothelial interactions by the activation and up-regulation of a variety of cell adhesion molecules (6). TNF- α also induces the binding of hyaluronan to CD44 expressed on peripheral blood monocytes (7). In this study, the human leukemic cell line SR91, which is positive for the myeloid-specific marker CD33 and negative for the CD2 and CD7 lymphoid markers, was used to investigate the effect of TNF- α on the adhesive state of CD44 (8).

Treatment of the SR91 cells with TNF- α (10 ng/ml) for 24 hours (9) resulted in increases in surface expression of the cell adhesion molecule, ICAM-1, and CD44 and induced the binding of fluoresceinated hyaluronan (FL-hyaluronan) (Fig. 1). FL-hyaluronan binding could be inhibited by IM7.8.1, a monoclonal antibody (mAb) to CD44, as well as by unlabeled hyaluronan (50 μ g/ml), indicating a specific interaction between CD44 and its ligand, hyaluronan. Stimulation of SR91 cells with interferon- γ (IFN- γ) (500 U/ml) also induced ICAM-1 expression but did not cause a significant increase in CD44 expression and did not induce FL-hyaluronan binding (Fig. 1); this result demonstrated the selectivity of the TNF- α response on CD44.

Given that TNF- α promotes leukocyte adhesion and migration at inflammatory sites, we sought to determine whether TNF- α induced the adhesion of SR91 cells to endothelial cells (10). TNF- α -stimulated SR91 cells, but not unstimulated or IFN- γ -treated cells, bound to a murine endothelial cell monolayer, SVEC4-10 (11) (Fig. 2). This adhesion was therefore not

dependent on the up-regulation of ICAM-1 and was not inhibited by an ICAM-1 mAb (12). The adhesion was CD44-dependent; preincubation of the TNF- α -treated SR91 cells with the CD44 mAb IM7.8.1 prevented the interaction. Preincubation of the murine SVEC4-10 monolayer with a murine-specific CD44 mAb, KM201, also prevented TNF- α -stimulated SR91 cells from binding to the endothelial cells, implying a homotypic, CD44-CD44-dependent interaction. Both CD44 antibodies block hyaluronan binding in human and murine cells, respectively, which suggests that the hyaluronan-binding regions of CD44 may be involved in the interaction. Preincubation of either cell line with hyaluronan had no significant inhibitory effect on adhesion (Fig. 2). However, pretreatment of either cell line with hyaluronidase abolished the interaction, suggesting a role for endogenous hyaluronan in bridging the CD44-CD44 interaction.

Previous work in fibroblasts had suggested a role for sulfation in regulating the hyaluronan binding of CD44. Inhibition of sulfation by chlorate, a potent inhibitor of adenosine triphosphate sulfurylase (13), prevented antibody-induced hyaluronan binding to CD44 (14). To elucidate the molecular mechanism by which TNF- α induces the adhesive ability of CD44 on SR91 cells, we first determined which forms of CD44 were present and whether TNF- α altered the sulfation pattern of CD44 (15). Only the 85-kD standard form of CD44 (CD44H) was detected in the SR91 cells after immunoprecipitation and blotting with IM7.8.1, and this form incorporated small amounts of [35 S]sulfate (Fig. 3). However, after TNF- α stimulation, the sulfation of CD44 was increased significantly, whereas CD44 expression was only slightly enhanced (Fig. 3). On average, sulfation was increased 4.5 ± 2.4 times ($n = 4$), whereas CD44 expression was increased only 1.9 ± 0.4 times ($n = 4$). Unlike TNF- α , stimulation of SR91 cells with IFN- γ had no effect on the sulfate incorporation of CD44 (Fig. 3). Thus,

the sulfation of CD44 was stimulated by TNF- α , but not by IFN- γ .

To determine whether the sulfation of CD44 was required for the induced adhesion of CD44 to hyaluronan and to endothelial cells, we incubated SR91 cells in the presence of 50 mM sodium chlorate, a potent sulfo-transferase inhibitor, during the TNF- α stimulation period (9). The TNF- α -induced FL-hyaluronan binding by CD44 was abrogated in the presence of sodium chlorate (Fig. 4A). Chlorate treatment of SR91 cells also abrogated the incorporation of [35 S]sulfate into CD44 (Fig. 4B). Chlorate treatment of SR91 cells did not prevent the increase in CD44 expression in response to TNF- α (Fig. 4, A and B) and did not affect cell viability, as assessed by trypan blue exclusion. This result indicated that an increase in CD44 expression alone could not account for the induction of CD44 adhesiveness. Treatment of SR91 cells with TNF- α in the presence of sodium chlorate also prevented the binding of SR91 cells to the SVEC4-10 monolayer (Fig. 2). Thus, TNF- α stimulated the sulfation of CD44, which converted CD44 from an inactive to an active cell adhesion molecule that could bind the extracellular matrix component hyaluronan and mediate leukocyte-endothelial cell adhesion.

Sulfation is an additional posttranslational modification that can regulate the adhesive function of CD44. It may provide one mechanism for regulating cell adhesion to vascular endothelial cells and may occur on tumor cells

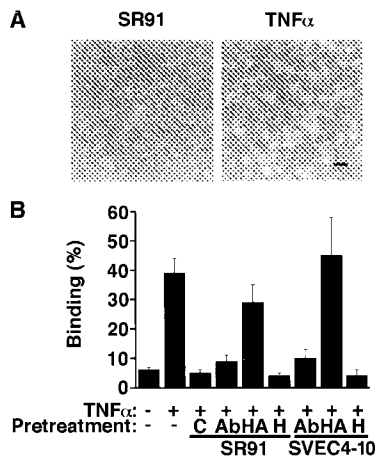


Fig. 2. Adhesion of SR91 cells to an SVEC4-10 endothelial cell monolayer. (A) Binding of unstimulated or TNF- α -stimulated SR91 cells to SVEC4-10 cells (scale bar, 100 μ m). (B) Percentage of 3×10^5 CFDA-labeled SR91 cells binding to the SVEC4-10 monolayer, untreated or pretreated as indicated, determined by fluorimetry. Pretreatment of SR91 cells or of SVEC4-10 was as indicated. C, chlorate; Ab, CD44 mAb (IM7.8.1 for SR91, KM201 for SVEC4-10 cells); HA, hyaluronan; H, hyaluronidase.

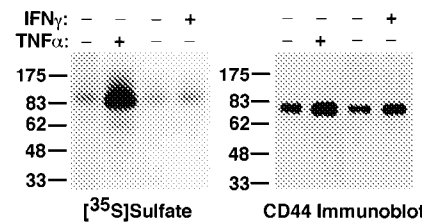


Fig. 3. Sulfate labeling of CD44. SR91 cells, either unstimulated (-) or stimulated with TNF- α or IFN- γ (+) (5×10^6 cells in each case), were labeled with [35 S]sulfate. One-quarter of the IM7.8.1-immunoprecipitated CD44 was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) under nonreducing conditions, then immunoblotted with the biotinylated CD44 mAb IM7.8.1; the remainder was resolved by SDS-PAGE under reducing conditions and exposed for autoradiography. Molecular mass markers are indicated (in kilodaltons).

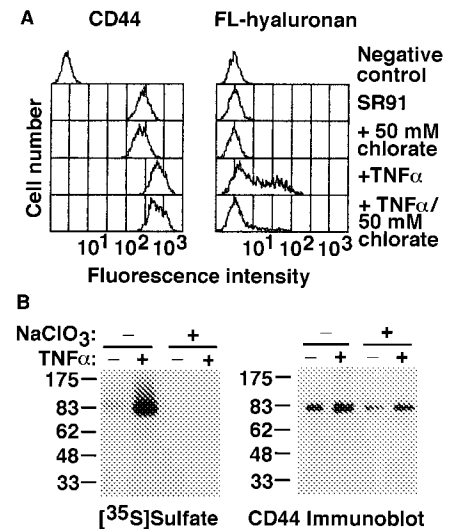


Fig. 4. Effect of chlorate on FL-hyaluronan binding ability and sulfation of CD44. Unstimulated and TNF- α -stimulated SR91 cells were incubated in the presence of 50 mM sodium chlorate. (A) CD44 expression and FL-hyaluronan binding profiles as determined by flow cytometry. Unlabeled SR91 cells were the negative control. (B) [35 S]sulfate-labeled IM7.8.1 immunoprecipitates of CD44 in the presence (+) or absence (-) of TNF- α or 50 mM chlorate. Molecular mass markers are indicated (in kilodaltons).

to facilitate their extravasation and metastasis. Keratan, heparan, chondroitin, carbohydrate, and tyrosine sulfate have been detected on alternatively spliced, higher molecular weight forms (116 to 200 kD) of CD44 (16, 17). In our study, the 85-kD form of CD44 was the major sulfated moiety. Preliminary data from sulfamino acid analysis provided no evidence for the tyrosine sulfation of CD44, whereas chemical or enzymatic removal of carbohydrate did provide evidence for carbohydrate sulfation (18). However, the precise identification of the sulfated residues and their location within CD44 remain to be determined. The selectin ligands are cell adhesion molecules involved in leukocyte-endothelial cell interactions that are also sulfated (19). These molecules mediate the initial rolling interactions between leukocytes and high-walled endothelial venules (HEVs) (20). Sulfation of two selectin ligands, GlyCAM-1 and CD34, occurs in HEVs on O-linked glycans and is required for high-affinity binding to L-selectin (19, 21). Tyrosine sulfation of the P-selectin ligand PSGL-1, present on leukocytes, has been shown to be important for high-affinity binding to P-selectin (22). Thus, the sulfation of cell adhesion molecules on HEVs and leukocytes may occur to facilitate leukocyte-endothelial cell interactions. The induction of CD44 sulfation by TNF- α provides one potential mechanism for regulating leukocyte adhesion during an inflammatory response.

fluorimeter (Millipore) or photographed (Zeiss Axio-phot). For inhibition studies, cells were pretreated with purified CD44 mAb IM7.8.1 [I. S. Trowbridge, J. Lesley, R. Schulte, R. Hyman, J. Trotter, *Immunogenetics* **15**, 299 (1982)], CD44 mAb KM201 [K. Miyake et al., *J. Exp. Med.* **171**, 477 (1990)], or ICAM-1 mAb (Cedarlane) in PBS (3 μ g/ml) with 2% FCS, rooster comb hyaluronan (50 μ g/ml; Sigma), or ovine hyaluronidase (5 μ g/ml; Calbiochem) and incubated for 20 min at 4°C after the labeling period. Cells were washed twice before mixing. CMFDA-labeled SR91 cells (3×10^5 ; 100% input cells) and wells containing the monolayer alone were used to determine 100% binding and background levels, respectively.

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23. Supported by research grants from the National Science and Engineering Research Council and the Arthritis Society of Canada. A.M. was supported by a studentship from the Heart and Stroke Foundation of British Columbia and Yukon. We thank H. Klingemann for providing the SR91 cell line.

28 July 1998; accepted 2 October 1998

Extended Life-Span and Stress Resistance in the *Drosophila* Mutant *methuselah*

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Toward a genetic dissection of the processes involved in aging, a screen for gene mutations that extend life-span in *Drosophila melanogaster* was performed. The mutant line *methuselah* (*mth*) displayed approximately 35 percent increase in average life-span and enhanced resistance to various forms of stress, including starvation, high temperature, and dietary paraquat, a free-radical generator. The *mth* gene predicted a protein with homology to several guanosine triphosphate-binding protein-coupled seven-transmembrane domain receptors. Thus, the organism may use signal transduction pathways to modulate stress response and life-span.

The effect of genes on life-span in *Drosophila* has been established by selective breeding (1). However, the participation of multiple genes with additive, quantitative effects can be difficult to unravel. A direct search for life-extension mutants could identify individual genes that regulate biological aging. Indeed, in the nematode *Caenorhabditis elegans*, several mutations, for example, *age-1*, *daf-2*, and *clk-1*, have been described that can increase the worm's life-span (2). The corresponding genes have been cloned and are involved in various aspects of development and metabolism (3, 4).

Life-span and stress response are closely associated. In *C. elegans*, the *age-1* mutant displays elevated resistance to thermal exposure (5) and to oxidative stress (6). In *Drosophila*,

laboratory stocks selected for postponed senescence also show increased tolerance to heat, starvation, desiccation, and oxidative damage (7–9). Tandem overexpression of Cu-Zn superoxide dismutase (SOD) and catalase genes in *Drosophila* increased life-span by 30% (10). Similar observations were made in flies expressing the human SOD1 transgene in motor neurons (11). However, the physiological and molecular events involved in life-span determination and stress resistance have remained largely elusive.

We generated a set of P-element insertion lines (12, 13) and screened them for ones that outlived a parent strain (*white*¹¹⁸). *methuselah* (*mth*) was isolated by its increase in life-span at 29°C. The life extension was confirmed at 25°C. At both temperatures, flies homozygous for the P-element lived, on the average, 35% longer than the parent strain (Fig. 1).

We then examined the ability of *mth* flies to resist stress. *mth* mutant flies were more resis-

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9. SR91 cells (10^6 cells/ml) were stimulated with TNF- α (10 ng/ml) or IFN- γ (500 U/ml; R&D Systems) in RPMI medium with 10% fetal calf serum (FCS) for 24 hours at 37°C in the presence of 5% CO₂. Sodium chlorate (50 mM; Sigma) was added concurrent with the cytokine treatment. Labeling of cells with mAbs or FL-hyaluronan for flow cytometry was as described (14). Cells were analyzed on a FACScan flow cytometer (Becton Dickinson) using Lysis II software.
10. Unstimulated and stimulated SR91 cells (10^6 cells/ml) were resuspended in 25 μ M CellTracker Green 5-chloromethylfluorescein diacetate (CMFDA; Molecular Probes) in RPMI medium for 30 min at 37°C, resuspended in RPMI with 10% FCS for 30 min at 37°C, and then washed. SR91 cells (3×10^5) were incubated with a confluent monolayer of SVEC4-10 cells in 0.5 ml of phosphate-buffered saline (PBS) with 2% FCS (in a 24-well plate) for 30 min at room temperature, then washed four times with PBS. Cells were fixed in 4% paraformaldehyde and analyzed on a Cytofluor Z300

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